

# A Polypeptidic Substance Found in Human Leukemic Urine Which Promotes Nonenzymic Acid Solubility of Nucleic Acids\*

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The existence of differences in nucleic acid metabolism among the various human leukemic states is illustrated by the patterns of incorporation of labeled precursors into the nucleic acids of the leukocytes (9, 10), the results of nucleic acid turnover studies (3), and the characteristic plasma and urinary uric acid levels (6, 8).

A further reflection of these differences in human leukemia might be found in the amounts of deoxyribonucleases (DNase I and DNase II) in the urine. These enzymes have been found (4) in precipitates produced by saturation of human urine with  $(\text{NH}_4)_2\text{SO}_4$ , and it has been demonstrated (5) that in the rat an altered physiological state (produced by x-radiation) increases the DNase activity of the urine.

In the method of assay of DNase usually employed (1), the acid-soluble products formed from deoxyribonucleic acid (DNA) when it is incubated with urine are determined. In the present report the use of this assay procedure revealed that DNA was rendered acid-soluble by human leukemic urine under conditions necessary for either the action of DNase I or of DNase II. On close examination it was found that this effect was in part nonenzymic. The nonenzymic action on DNA could be attributed to the presence in the leukemic urine of a heat-stable, nondialyzable substance which is inactivated by incubation with proteolytic enzymes.

## MATERIALS AND METHODS

*Urine collection.*—Twenty-four-hour urine specimens from normal subjects and from patients

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with leukemia and other malignancies were collected under toluene, cooled to 4° C., and filtered. A sample of each urine was dialyzed (Visking casing) against cold running tap water in a 4° C. cold room overnight, followed by distilled water until the dialysate was free of chloride. The samples were stored in the frozen state after dialysis.

*Assay for DNase I and DNase II in urine.*—The methods of Allfrey and Mirsky (1), as modified by Kowlessar *et al.* (5), with the use of calf thymus DNA (Worthington Laboratories, Freehold, New Jersey), were used. To the standard substrate solutions, 0.5 ml. of water or urine was added, and the mixtures were incubated for varying lengths of time at 37° C. Trichloroacetic acid (TCA) was added to a concentration of 9 per cent. After centrifugation at 2° C. and  $800 \times g$  for 20 minutes, the diphenylamine reaction (2) was applied to the supernatant fluids. The amount of DNA solubilized was determined by means of a standard curve; corrections were made for urine and substrate blanks.

*Turbidimetry.*—One ml. of 38 per cent TCA was added to 4 ml. of an aqueous solution of herring-sperm DNA (California Foundation for Biochemical Research). The optical density of the resulting suspension, which reached a maximum value in a few minutes, was measured in the Klett-Summer-son photoelectric colorimeter equipped with filter 66 or in the Beckman Spectrophotometer at 660  $m\mu$ . Maximal optical densities were directly proportional to the amount of DNA up to 4 mg. The prevention of acid precipitation of DNA by dialyzed urine or solutions of solids prepared from such urine was studied, with appropriate water controls.

*Fractional precipitation of urine with ethanol.*—Approximately 1500 ml. of dialyzed urine was concentrated, by boiling, to 1 liter. Further concentration was affected by evaporation in shallow

pans on a steam bath with a current of air passing over the surface. The temperature was maintained between 50 and 60° C. When the volume was about 100 ml., the material was centrifuged, the sediment washed with a little water, the wash solution combined with the supernatant fluid, and the sediment discarded. Ethanol was added to the solution to a concentration of 30–40 per cent. After being cooled for 1 hour at –10° C., the precipitate which appeared from leukemic urine concentrates exhibited slight activity in the turbidimetric test system and was discarded. The ethanol concentration was brought to 65 per cent, and after being cooled for 1 hour at –10° C., the precipitate was collected, washed successively with 80 per cent ethanol, absolute ethanol, ethanol-ether (1:1), ether, and air-dried. Solids prepared in this fashion from urines of five leukemic patients (two acute myeloblastic, one acute lymphoblastic, one acute

tions, 40–60 per cent of the DNA was nonprecipitable by TCA after 5 minutes in both the DNase I and II assays. Further incubation yielded additional acid-soluble products of DNA under DNase I conditions, but DNase II activity was very low or absent. Prior heating of leukemic urine did not abolish the effect of short-term incubation but prevented further degradation of DNA in the DNase I assay. A further study of the influence of time of incubation of DNA with leukemic urine in the DNase II test system revealed that as much DNA was rendered acid-soluble in 1 minute as in 120 minutes.

When dialyzed leukemic urine was mixed with an aqueous solution of DNA at room temperature and TCA added immediately, there resulted a decreased precipitation of DNA as measured turbidimetrically. Chart 1 compares a leukemic with normal urine, both of which had been concen-

TABLE 1  
EFFECTS OF DIALYZED NORMAL AND LEUKEMIC URINE ON CALF THYMUS DNA  
Expressed as per cent of DNA not precipitated by TCA

INCUBATION CONDITIONS		NORMAL		CHRONIC LYMPHOCYTIC LEUKEMIA		ACUTE MYELOBLASTIC LEUKEMIA*	
		5	120	Time of incubation (min.)		5	120
				5	120		
Unheated urine	DNase I	0	36	43	82	60	87
	DNase II	0	1	43	50	63	60
Heated urine	DNase I	0	1	53	56	38	39
	DNase II	1	2	53	55	38	39

\* The urine of this patient was diluted 1:3 with water after being heated.

monoblastic, one chronic lymphocytic) were active in the turbidimetric test system. Supernatant fluids from the 65 per cent ethanol precipitation step yielded inactive material at higher concentrations of ethanol. The urine from four normal subjects yielded inactive solids between ethanol concentrations of 35 and 65 per cent. The amount of protein in these preparations was determined by a colorimetric biuret method and by the method of Lowry *et al.* (7), with crystalline ovalbumin used as the standard.

## RESULTS AND DISCUSSION

The effects of incubating DNA with dialyzed normal or leukemic urine are illustrated by the data recorded in Table 1. Under conditions for the action of DNase I, normal urine did not produce acid-soluble products from DNA in 5 minutes but degraded approximately 36 per cent of the DNA in 120 minutes. DNase II activity was not detected. Heating normal urine (1 hour, boiling water bath) abolished DNase I activity. When leukemic urine was tested under the same condi-

trated 13-fold by heat evaporation at atmospheric pressure after dialysis. Mixing normal urine with leukemic urine did not abolish the effect of the latter. When the Dische reaction and turbidimetry and either calf-thymus or herring-sperm DNA was used, it was found that varying the pH of the solution of leukemic urine and DNA from 3.6 to 9 was without influence on the amount of DNA which remained soluble when TCA was added 1 minute after mixing.

The dialyzed urines of sixteen patients in a group of twenty with various forms of leukemia have produced this effect on DNA. This group of eight females and twelve males included four patients with chronic myelocytic leukemia, eight with chronic lymphocytic leukemia, three with acute lymphoblastic leukemia, four with acute myeloblastic leukemia (including one case of erythroleukemia), and one with acute monoblastic leukemia. The inactive urines came from two patients with chronic lymphocytic leukemia, one with chronic myelocytic leukemia, and one with acute lymphoblastic leukemia. DNase II was not

detectable in active urines from five of these patients. In earlier experiments, the undialyzed urines of twenty of a group of 24 untreated patients solubilized over 80 per cent of the calf-thymus DNA in the standard DNase II assay system in 3 hours. It is most probable that these urines did not exhibit DNase II activity and that acid-solubilization of DNA occurred immediately.

Leukemic urines were active while the patients were in relapse of their disease and remained active during depression of the bone marrow and blood elements following chemotherapy. The rapid destruction of a large number of leukemic cells by therapy, decrease in the sizes of the spleen and

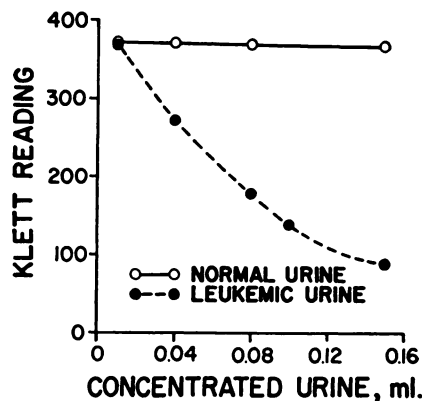


CHART 1.—Effects of dialyzed concentrated urines from a patient with acute myeloblastic leukemia and from a normal subject on the precipitation of DNA by TCA.

lymph nodes and increased urinary excretion of uric acid was not accompanied by the appearance of the activity in the urines which had been initially devoid of that activity. None of the patients had overt renal function impairment or proteinuria (except for a trace of protein in the urine of a few subjects). In two subjects, one with acute myeloblastic leukemia and one with acute monoblastic leukemia, the urine remained active while the patients were solely on intravenous therapy and were not taking anything orally.

The urines of eighteen of 22 nonleukemic patients with advanced cancer, frequently accompanied by general metastases and invasion of the bone marrow, were inactive. The urines of 40 normal subjects were inactive.

Heating dialyzed urine for 5 minutes at 98° C. enhanced activity only in the case of leukemic urine (Table 2). Further heating for 1.5 hours did not result in loss of activity. The time-independent character of the effect, the absence of a pH optimum for its manifestation and failure of inactivation of leukemic urine by long periods of heating at

98° C. indicate that, whatever the precise nature of the effect on DNA, the responsible factor is not a DNase. The factor in leukemic urine was completely inactivated in 0.1 N NaOH in 15 minutes at 98° C. and in 0.5 N HCl in 45 minutes at the same temperature.

Evidence that the nondialyzable factor is most probably polypeptidic in nature was obtained in the following manner. Dialyzed urines from four leukemic patients were concentrated five- to tenfold by evaporation on a steam bath at atmospheric pressure. The urines were then diluted with water to the extent that 0.2 ml. of these solutions would prevent the precipitation of not more than 1 mg. of calf-thymus DNA in a final volume of 2 ml. containing 9 per cent TCA. Samples of the urines were then adjusted to pH 2.5, and crystalline pepsin was added to a concentration of 50  $\mu$ g/ml. Aliquots were immediately withdrawn for test as above and the remainder incubated at

TABLE 2  
ACTIVATION OF DIALYZED LEUKEMIC URINE BY HEAT  
Detected turbidimetrically\*

Diagnosis	Unheated urine Klett reading	Heated urine Klett reading
Normal	205	195
Normal	192	186
Normal	194	189
Normal	197	195
Acute myeloblastic leukemia	78	48
Erythroleukemia	118	27
Chronic lymphocytic leukemia	85	50
Chronic myelocytic leukemia	100	72

\* One minute after the addition of 0.5 ml. of urine to 2 mg. of herring-sperm DNA contained in 4 ml. of water, 1 ml. of 38 per cent TCA was added. Turbidity was recorded 5 minutes later in the Klett-Summerson colorimeter with filter 66. Controls with water in place of urine gave readings of 197–201.

37° C. for 1 hour. Urines at pH 2.5 incubated without pepsin served as controls. Equal aliquots of the incubated samples were then added to calf-thymus DNA solutions, followed by TCA, diluted with water, and the turbidities recorded. In all cases TCA was added 1 minute after mixing. Duplicate samples were centrifuged, and the diphenylamine reaction (2) was applied to the supernatant solutions. The results given in Table 3 demonstrate complete inactivation of the leukemic urine by pepsin.

The use of pepsin also permitted the demonstration that the effect on DNA was reversible. Herring-sperm DNA, 1 mg/ml in dilute HCl, pH 2.5, was supplemented with either concentrated leukemic urine, concentrated leukemic urine plus pepsin, or water plus pepsin. Aliquots of these mixtures were withdrawn immediately after mixing, TCA added, and turbidities recorded. The remain-

ing solutions were incubated at 37° C., aliquots withdrawn after 10 and 25 minutes, TCA added, and turbidities again recorded. The data in Table 4 show that the acid precipitability of the DNA incubated with pepsin or with urine remained constant. The DNA incubated with urine plus pepsin became increasingly less acid-soluble, full restoration of precipitability occurring after 25 minutes. This reversal is indicative of the nonenzymic effect of the leukemic factor on DNA. If an enzymic degradation of the DNA had taken place it would not be expected that incubation with pepsin would cause recombination of split products of the DNA to produce an acid-insoluble polymer.

was abolished. Incubation under comparable conditions but in the absence of the proteolytic enzymes resulted in no loss of activity. The amounts of active precipitates obtained from the urine of five leukemic patients ranged from 0.2 to 1.4 gm/l. The amounts of material precipitated at the same alcohol concentration from the urine of four normal subjects ranged from 0.4 to 0.5 gm/l. When analyzed for protein content by the method of Lowry *et al.* (7), the solids from leukemic urine were found to consist of 20-98 per cent protein, while the solids obtained from normal urines contained no more than 6 per cent protein. In attempting to analyze for protein by a conventional

TABLE 3  
EFFECT OF PEPSIN ON LEUKEMIC URINE

DIAGNOSIS	ABSORBANCE IN DISCHEM REACTION			TURBIDITY, KLETT READINGS		
	Control	Pepsin	Time of incubation (min.)	Control	Pepsin	60
	60	0		60	0	
Chronic myelocytic leukemia	0.415	0.415	0.025	11	8	110
Acute myeloblastic leukemia	0.400	0.480	0.060	14	20	113
Acute myeloblastic leukemia	0.415	0.430	0.010	45	38	119
Erythroleukemia	0.400	0.350	0.020	50	50	118
Normal urine	0.005		0.010	114	117	117
Water	0.010		0.010	117	118	115

TABLE 4  
CONVERSION BY PEPSIN OF ACID-SOLUBLE DNA  
IN LEUKEMIC URINE TO ACID-INSOLUBLE DNA

Incubation time (min.)	Detected turbidimetrically		
	DNA + water + pepsin	DNA + urine + water Klett readings	DNA + urine + pepsin
0	350	147	151
10	350	150	280
25	335	150	335

Active solids were obtained from concentrated dialyzed leukemic urine by precipitation with ethanol. In Table 5 are shown their effects on calf-thymus DNA and calf liver ribonucleic acid (RNA).<sup>1</sup> Prevention of precipitation is not limited to DNA. The leukemic material was not inactivated by contact with 38 per cent TCA for 1 minute at room temperature. Solids prepared in comparable fashion from normal urine were inactive. When aqueous solutions containing 1.5 mg/ml of the leukemic solids were incubated for 1 hour at 37° C. with pepsin (50 µg/ml, pH 2.5), chymotrypsin (20 µg/ml, pH 7.5), and trypsin (50 µg/ml, pH 7.5),<sup>2</sup> the activity toward both DNA and RNA

biuret method, difficulty was encountered because of high blank readings in the absence of copper. Corrections for these blanks yielded protein values of the order of 50 per cent lower than those obtained by the method of Lowry *et al.* (7). The results obtained by both methods, however, revealed that considerably more protein was present in the leukemic materials than in those obtained from the normal subjects. Preliminary results of amino acid analysis by one-dimensional paper chromatography of 6 N HCl hydrolysates of preparations from two leukemic subjects revealed the presence of at least eleven ninhydrin-positive compounds in each.

The lack of knowledge of the precise chemical nature of the leukemic urinary substance, which is undoubtedly polypeptidic, prevents cogent discussion concerning its physiological significance in leukemia. No information is available as to whether it plays a role in nucleic acid metabolism. Some unpublished experiments in this laboratory demonstrated that the substance is not an inhibitor of crystalline pancreatic DNase or of DNase II of human leukemic leukocytes. The nature of the combination of the leukemic material with nucleic acids *in vitro* is not known. The solubilization of these compounds in TCA points to the formation of nucleic acid-polypeptide complexes in which the

<sup>1</sup> We are indebted to Dr. E. L. Grinnan for a sample of calf-liver RNA.

<sup>2</sup> The crystalline enzymes were obtained from Worthington Biochemical Corporation, Freehold, N.J.

components are tightly bound. Possibly these complexes assume a net positive charge which prevents aggregation in the acid solution.

### SUMMARY

The urine of patients with various forms of leukemia contains a nondialyzable, heat-stable substance which prevents the precipitation of nucleic acids by trichloroacetic acid. The leukemic urinary

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TABLE 5  
EFFECT OF URINARY SOLIDS ON ACID-PRECIPITABILITY OF NUCLEIC ACIDS  
Detected turbidimetrically

DIAGNOSIS	AMT. OF SOLID ( $\mu\text{g.}$ )	DNA	ORDER OF ADDITION		RNA
			A*	B†	
			DNA	DNA	
	0	0.278	0.297	0.280	0.310
Acute myeloblastic leukemia	50	0.094	0.145	0.104	0.147
	150		0.067		
Acute monoblastic leukemia	50	0.148	0.153	0.174	0.150
	150		0.058		
Acute myeloblastic leukemia	50	0.170	0.188	0.217	0.173
	150		0.093		
Chronic lymphocytic leukemia	50	0.106	0.168	0.136	0.143
	150		0.082		
Acute lymphoblastic leukemia	50	0.049	0.133	0.076	0.103
	150		0.051		
Normal	50	0.239	0.298	0.293	0.305
	150		0.292		
	250		0.294		
Normal	50	0.273	0.298	0.296	0.296
	150		0.292		
	250		0.283		

\* 500  $\mu\text{g.}$  of nucleic acid in 4.0 ml. of  $\text{H}_2\text{O}$ +0.01 or 0.03 ml. of aqueous solution of urinary solid; 1.0 ml. of 38 per cent TCA added 1 min. after mixing. Maximum turbidity recorded.

† 0.01 or 0.03 ml. of aqueous solution of urinary solid +1.0 ml. 38 per cent TCA; 500  $\mu\text{g.}$  of nucleic acid in 4.0 ml. of  $\text{H}_2\text{O}$  added 1 min. after mixing. Maximum turbidity recorded.

substance is polypeptidic but is not a deoxyribonuclease. The substance can be isolated as a crude solid by precipitation with ethanol from concentrated leukemic urine. Substances with similar activity are absent from normal human urine and are most probably excreted rarely by patients with nonleukemic malignancies.

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